Associations of Mitochondrial and Nuclear Mitochondrial Variants and Genes with Seven Metabolic Traits

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ARTICLE

Associations of Mitochondrial and Nuclear Mitochondrial Variants and Genes with Seven Metabolic Traits

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Mitochondria (MT), the major site of cellular energy production, are under dual genetic control by 37 mitochondrial DNA (mtDNA) genes and numerous nuclear genes (MT-nDNA). In the CHARGEmtDNA+ Consortium, we studied genetic associations of mtDNA and MT-nDNA associations with body mass index (BMI), waist-hip-ratio (WHR), glucose, insulin, HOMA-B, HOMA-IR, and HbA1c. This 45-cohort collaboration comprised 70,775 (insulin) to 170,202 (BMI) pan-ancestry individuals. Validation and imputation of mtDNA variants was followed by single-variant and gene-based association testing. We report two significant common variants, one in *MT-ATP6* associated ($p \leq 5E{-}04$) with WHR and one in the *D-loop* with glucose. Five rare variants in *MT-ATP6*, *MT-ND5*, and *MT-ND6* associated with BMI, WHR, or insulin. Gene-based meta-analysis identified *MT-ND3* associated with BMI ($p \leq 1E{-}03$). We considered 2,282 MT-nDNA candidate gene associations compiled from online summary results for our traits (20 unique studies with 31 dataset consortia's genome-wide associations [GWASs]). Of these, 109 genes associated ($p \leq 1E{-}06$) with at least 1 of our 7 traits. We assessed regulatory features of variants in the 109 genes, *cis*- and *trans*-gene expression regulation, and performed enrichment and protein-protein interactions analyses. Of the identified mtDNA and MT-nDNA genes, 79 associated with adipose measures, 49 with glucose/insulin, 13 with risk for type 2 diabetes, and 18 with cardiovascular disease, indicating for pleiotropic effects with health implications. Additionally, 21 genes related to cholesterol, suggesting additional important roles for the genes identified. Our results suggest that mtDNA and MT-nDNA genes and variants reported make important contributions to glucose and insulin metabolism, adipocyte regulation, diabetes, and cardiovascular disease.

Introduction

Mitochondria (MT) are double membraned organelles that generate the majority of cellular adenosine triphosphate (ATP) and metabolites and play a central role in human health.[1] MT reside within cells and contain a separate maternally inherited[2–5] ~16.6 kb circular mtDNA genome, consisting of 37 genes (Figure 1). The mtDNA encodes 13 core catalytic peptides that form the oxidative phosphorylation complexes (I, III-V) as well as the machinery needed

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to translate these peptides consisting of 22 transport RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs). In addition, a noncoding sequence, known as the displacement loop (*D-loop*), encompasses the replication origin(s) and promoters for mtDNA.

MT function is also dependent upon many nuclear genes that encode proteins involved in mtDNA transcription, replication, cell apoptosis and mitophagy, nucleotide biosynthesis, metabolism, and iron and calcium homeostasis. Here we evaluate 2,282 candidate nuclear genes that also may contribute to MT function. We refer to these genes as "MT-nDNA candidates," curated for this study based upon protein co-localization within the MT and text mining.[6–8] Unlike mtDNA-encoded proteins that are transcribed and translated within MT, MT-nDNA genes are translated in the cytoplasm and their proteins are transported into MT through porins or complexes such as translocases.

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The dual genomic origin (mtDNA and MT-nDNA) of MT components and the fact that mtDNA has a much higher mutation rate compared to the nuclear genome[9,10] fuels our hypothesis that MT variants influence individual predisposition to complex diseases.[11,12] Because of heteroplasmy, i.e., the coexistence of mutated and wild-type mtDNA in the same cell, mutations in mtDNA may not result in disease until the copy number of mtDNA carrying the mutation relative to the wild-type mtDNA in the relevant tissue(s) exceeds a threshold.[12] The mtDNA copy number may serve as an indicator of MT function[13] and was recently shown to be associated with overall mortality[14,15] and various metabolic-related diseases, including cardiovascular disease (CVD).[16–18] Studies have also shown that mitochondria have an important influence on multiple cardiovascular disease risk factors. For example, obesity impairs MT biogenesis.[19–21] MT dysfunction has been also implicated in the development of insulin resistance.[22–24] Thus, our study prioritized obesity and insulin resistance pathways to CVD.

In our study we analyzed the dual genomic influence of MT function on seven important metabolic traits (body mass index [BMI], waist-hip ratio [WHR], fasting glucose, fasting insulin, HOMA-B, HOMA-IR, and HbA1c) as major risk factors of cardiovascular disease (CVD) in participants of the CHARGEmtDNA+ 45 participating cohorts. The mtDNA associations were evaluated at both the single nucleotide variant (SNV) and at the gene-based levels for rare alleles (minor allele frequency [MAF] < 1%). The MT-nDNA candidates were studied for function, gene expression regulation, enrichment of pathways, and protein interactions. The overall goal was to illuminate MT-functioning and MT-disease causative variants.

Subjects and METHODS

CHARGE Cohorts

There were 45 participating cohorts with a total of 196,967 individuals (166,423 individuals were of the European [EA], 15,745 of African [AA], 1,567 of Asian [ASA], 11,307 of Hispanic/Latinos [HA], and 1,925 of Brazilian ancestry) who had at least one of the seven traits. CHARGE cohort summary descriptions are available online (see Supplemental Study Descriptions). The number of samples available from all cohorts differed by phenotype (Table S1). The resulting samples were smaller within each cohort when association tests were performed (by sub-setting individuals with genotyping available) to a total of 170,202 for BMI, 155,396 for WHR, 79,906K for fasting glucose, 70,778 for fasting insulin, 69,845 for HOMA-B, 69,926 HOMA-IR, and 101,218 for HbA1c. We use the acronym PA to refer to pan-ancestry. The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and that proper informed consent was obtained.

Trait Harmonization

A total of seven traits were studied: BMI (kg/m^2), WHR (waist-hip ratio), fasting plasma glucose (mg/dL), fasting insulin (μIUmL),

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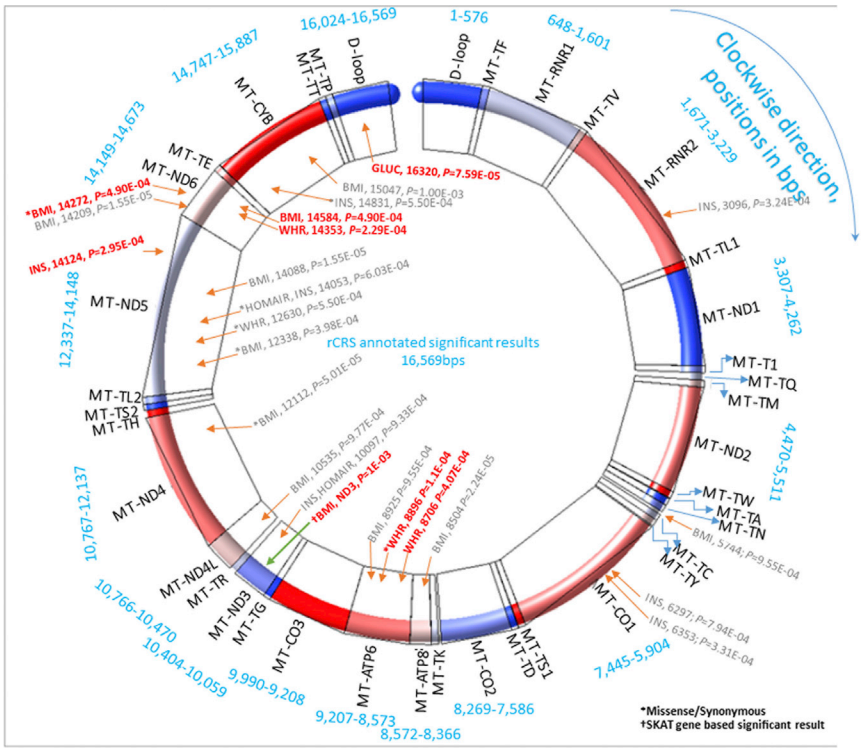

Figure 1. Graphical Presentation of Single mtDNA- and Gene-Based Association Meta-Analysis Results

Significant associations (trait, MT position, and p value) at liberal threshold ($p \leq 1E{-}03$) are annotated with red arrows and gray text for single SNV associations and with green arrow for gene-based association, and text in red color represents SNVs that passed the threshold $p \leq 5E{-}04$. The asterisk (*) marks missense or synonymous variants; a dagger (†) marks a SKAT gene-based significant association. The mtDNA is shown as one single molecule, by merging all genes from heavy and light MT strands. For visibility, gene colors rotate clockwise from first in blue in the *D-loop* to up to six coloring groups finishing in red at *MT-TL1*, and then recycling again from the first blue color at *MT-ND1*. The full information is summarized in Tables 1, 2, 3, and S2.a-S2.b. In blue text are shown bps-positions of 13 MT-coding genes (*MT-ND1*, *MT-ND2*, *MT-CO1*, *MT-CO2*, *MT-ATP8*, *MT-ATP6*, *MT-CO3*, *MT-ND3*, *MT-ND4L*, *MT-ND4*, *MT-ND5*, *MT-ND6*, *MT-CYB*), 2 MT-ribosomal RNAs (MT-RNR1 [12S RNA] and MT-RNR2 [16S RNA]), and 22 transport RNAs (*MT-TF*, *TV*, *TL1*, *T1*, *TQ*, *TM*, *TW*, *TA*, *TN*, *TC*, *TY*, *TS1*, *TD*, *TK*, *TG*, *TR*, *TH*, *TS2*, *TL2*, *TE*, *TT*, *TP*).

HOMA-B, HOMA-IR, and glycated hemoglobin (HbA1c, %) indicative of chronically elevated glucose levels. The homeostasis model assessment was based on plasma levels of fasting glucose and fasting insulin. HOMA-B is an indicator of beta-cell function calculated as HOMA-B = (360 × fasting insulin) / (fasting glucose − 60). HOMA-IR is an indicator of insulin resistance calculated as HOMA-IR = (fasting glucose × fasting insulin) / 405. Glucose and insulin measures were required to have been measured with a minimum fasting time of 8 hr; otherwise, the measure was set to missing. Each study was responsible for ensuring that each trait was normally distributed. Natural-log transformation was implemented for insulin, power transformation was performed when no other solutions were available, and inverse normal transformation was used as a last resort for BMI[25] (details in the Supplemental Material and Methods, section 1). The association test analysis was restricted to non-diabetic participants when studying glycemic traits, by setting values of glucose, insulin, and HbA1c to missing for T2D individuals with HbA1c > 6.5 or with fasting glucose > 126 mg/dL or using T2D medications. The participating cohorts were required to have at least one of the seven studied metabolic traits.

Preparation of Traits for Analyses

All cohorts used the same units for all traits for the raw measures. We observed differences in the raw measures of traits (Table S1). Thus, to harmonize results, all participating cohorts normalized trait distributions and produced standardized residuals, which are unit-less for each trait. Statistical regression via R / SAS programming languages was used to produce residuals fitting the following model:

$$\text{trait} = \text{age} + \text{age}^2 + \text{sex} + \text{PCs} + \text{other study specific covariates (e.g., study center, etc)}$$

Age^2 was used to remove any age-quadratic trend from the response variable, and principal components (PCs) represent one or more genotype principal components, to minimize population substructure. The standardized residuals from this regression were the final response variables for each trait, used in the association tests with imputed dosages of mtDNA.

mtDNA Variant Harmonization

All mtDNA variants from each array (Table S14) were annotated to the nucleotide position according to the rCRS of the human mitochondrial DNA prior to analyses (see Web Resources). The probes used for each microarray were obtained from the manufacturer or dbSNP and aligned to the rCRS using Geneious 8.1.[26] All probes were also submitted through the standard nucleotide basic local alignment search tool (BLAST) to ensure the probes bound with high specificity (≥90% identity) to the mitochondrial genome. In order to limit any potential binding to nuclear DNA segments, probes that bound to nuclear chromosomes with ≥80% matching were excluded from all analyses. The full lists of the validated mtDNA variants are available in the Supplemental Material and Methods, section 2 and Tables S15–S28.

mtDNA Imputation

The preparation of mtDNA data for imputation was done with PLINK.[27,28] When preparing data for imputation, a few heterozygotes that may have existed in the mtDNA genotypes were set to missing via PLINK. The prephasing of mtDNA scaffold haplotypes at the cohort level was done with SHAPEIT2 for the full mtDNA (see Web Resources).[29] SHAPEIT2 helped to have the mtDNA genotypes of a cohort in the right format to be used in the imputation pipeline with IMPUTE2. SHAPEIT2 was also used for QC checks, such as evaluating missingness in markers. In order to combine

analyses across different genotyping platforms, we performed imputation based on MT-1000G (see Supplemental Material and Methods, section 3). Imputations of mtDNA variants were implemented in IMPUTE2 at the cohort level in a window that included the full MT-chromosome (see Web Resources).[30] Recoding of genotype probabilities into dosages was implemented via FCGENE (see Web Resources). Our reference MT-chromosome panel consisted of the 1000G data (PHASE 3, v.5) (see Web Resources). These data were based on sequence freeze and alignments, using only Illumina platform sequences and only sequences with read lengths of 70 bps or greater. There were 3,617 mtDNA markers in 1000G. The 1000G included 2,504 individuals representing 26 sampled populations (the "Cosmopolitan" reference panel).[31] After imputation, cohorts had differing number of SNVs, up to 3,832 SNVs for BMI, 3,829 for WHR, 3,809 for glucose, 3,801 for insulin, 3,801 for HOMA-B, 3,801 for HOMA-IR, and up to 3,744 for HbA1c. Although the imputation quality was good for most of cohorts (see imputation accuracy in Table S14), a great number of the SNVs were filtered for INFO (imputation quality) < 0.30, monomorphic or very rare (MAF < 0.0001) at the cohort level before association tests. Other QC were genotype call per cell had to be $p \geq 0.90$, a marker was dropped if its missing rate was >0.05 and MAC had to be ≥ 5, (which if it was autosomal could have corresponded to MAC ≥ 10). The MAC was calculated for haplotypes as MAF*N. (See Supplemental Material and Methods, section 3 for a more detailed methodology.) After mtDNA imputation, before performing statistical associations, we set any heteroplasmic mutations to missing at the cohort level data. Details of QC via R Package: EasyQC (V10.0)[32] (see Web Resources) and our internal programs and of the imputation steps can be found in the Supplemental Material and Methods, section 3. All filters implemented at the cohort level created non-uniform contributions of all cohorts in the meta-analysis. Many of the mtDNA variants were rare and per trait meta-analyses QQ-plots often showed an underestimation compared to expected association results (Figures S1–S7). The rarity of alleles was accompanied with lower quality of imputation and accordingly many of them were removed before any meta-analysis. Most studies had good imputation quality, given the rarity of mtDNA variants.

Variants MT-14272 and MT-14584 are opposite C/T versus T/C, but in full LD, while they are rare. The typical assumption is that rare variants are not in LD, but that does not have to hold for mtDNA. This is one more observation that the rare association findings in this study represent potential associations, until replicated from other future studies.

mtDNA Association Statistical Analyses

An additive genetic model was applied in association analyses using both self-developed regression models (the linear or linear mixed models written in R programming) and the SKAT (the prepScores() function in seqMeta R package: seqMeta package**:** Meta-Analysis of Region-Based Tests of Rare DNA Variants approaches[33] (see Web Resources). Familial and maternal correlation structures[34] were accounted for in the analysis of family data. Details of statistical association models 1–4 are described in the Supplemental Material and Methods, section 4.

mtDNA Meta-Analyses

Single-variant fixed-effects meta-analyses were conducted with METAL,[35] and gene-based associations were performed in seqMeta (details in the Supplemental Material and Methods, sections 5–7) (Tables 1, 2, 3, S2.a, S2.b, and S13). The average allele frequency from Metal is an average of allele frequencies for a particular marker as contributed from each study and weighted by each study's sample size as follows: $Freq1WeightAve = \sum_{k=1}^{s} w_k f_k / \sum_{k=1}^{s} w_k$, where k represents the indexing of contributing studies, w_k is the sample size (number of individuals) per study, and f_k is the coded allele frequency for a particular marker from a particular study. Our working group conducted an internal permutation test[36] using ARIC study mtDNA data and determined that 49 independent mitochondrial variants represented an estimate of the number of independent genetic effects for mtDNA. Thus, 49 was used as a denominator for producing the Bonferroni corrected p significance threshold $\leq 0.05/49 \leq 1E{-}03$, a threshold for common variants (MAF $\geq 1\%$), but possibly liberal for rare variants (MAF $< 1\%$) (Table 1). Furthermore, we considered $p \leq 0.05/(49 \times 7 \text{ traits}) \leq 1.5E{-}04$ as a conservative threshold when accounting for seven traits tested. We settled for a Bonferroni-threshold $p \leq 0.05/(49 \times 2 \text{ domains}) \leq 5E{-}04$, because the traits within a domain adipose/obesity (BMI and WHR) and glucose metabolism (glucose, insulin, HOMA-B, HOMA-IR, and HbA1c) are correlated and represent two domains. Because mtDNA is a small genome, the distributions of mtDNA QQ-plots with the existing samples do not always behave similarly to those observed for larger nuclear GWASs. While mtDNA variants MAF $\geq$ 1% formed relatively good QQ-plot distributions, the rare variants (MAF $< 1\%$) were sometimes distributed with some deviation in the start (bottom-left) of the QQ-plot, as a result of meta-results in very rare alleles. Thus, the quality control of mtDNA QQ-plots were implemented by filtering at different rare MAF levels. We concluded that with the existing mtDNA meta-analysis results, QQ-plots with MAF $> 0.8\%$ were acceptable. Thus, out of 23 SNVs nominally ($p \leq 1E{-}03$) associated with 6 metabolic traits (Figure 1), by adding the filter of MAC ≥ 5 for each cohort, then 7 variants passed $p \leq 5E{-}04$ Bonferroni threshold.

For the gene-based analysis, we used Burden tests which combine the contributions of rare genetic variants within a gene region. Such tests assume similar directionality and effect sizes for each variant.[33] In contrast, the sequence kernel association test (SKAT) for unrelated or family-based studies is an efficient regression-based approach for rare genetic variant analysis.[33,37,38] The meta-analyses were performed using seqMeta (see Web Resources). The gene-based Bonferroni p value was calculated as $p \leq 0.05/$ (37 mtDNA genes) $\leq 1E{-}03$.

Identification of MT-nDNA Candidate Genes

We established a list of 2,282 MT-nDNA candidate genes from three sources: (1) Mito Carta 2.0,[6,39] (2) Literature Lab (Acumenta Biotech), and (3) MitoMiner (4.0)[7] (see also Supplemental Material and Methods, section 7). We used the two separate sets of human genes and mouse ortholog genes from MitoCarta. We used Literature Lab to perform a literature search and identified 36 terms based on MeSH mitochondria. From each of the 36 terms (Table S29), we selected only the upper quartile list of genes from a Log Probability Function - scoring distribution. Each term was tested for association in overlapping with genes in pathway analysis (Figure S15). We conditioned this list with only genes from human nomenclature and accepted only genes that had more than 15 abstracts cited per selected gene (see Supplemental Material and Methods section 7).

Using MitoMiner we identified additional MT-nDNA candidate genes. They were filtered with a MT-MitoMiner index ≥ 0.70,

Table 1. mtDNA Variants Associated with BMI, WHR, Glucose, Insulin, HOMA-B, HOMA-IR, and HbA1c METAL Meta-Analysis Single-Variant Results

No	Pos	rsID	Gene	Annotation	Trait	A1/2	Freq1 WeightAve	FreqSE	MinFreq	MaxFreq	MAF	MAC	β(SE)	p	Dir	Het-p	N
Meta-analysis Level Results Selected with MAF of Weighted Allele Average Frequency > 1% and Passing Meta-p-Threshold ≤ 5E−04																	
1	8706	–	MT-ATP6	–	WHR	A/G	0.9676	0.0295	0.9135	0.9974	0.0324	834	−0.13 (0.04)	4.1E−04	----+	9.44E−02	25,748
2	16320	rs62581338	D-loop	–	GLUC	T/C	0.0158	0.0954	0.0028	0.2439	0.0158	301	−0.21 (0.05)	7.6E−05	--+--	1.77E−01	19,046
Meta-analysis Level Results Selected with MAF of Weighted Allele Average Frequency < 1% and Passing Meta-p-Threshold ≤ 5E−04																	
1	8896	rs202120082	MT-ATP6	missense	WHR	A/G	0.0038	0.0045	0.0003	0.0121	0.0038	134	0.30 (0.08)	1.12E−04	++++	8.80E−01	34,959
2	14124	–	MT-ND5	–	INS	T/C	0.0035	0.0029	0.0022	0.0087	0.0035	21	0.57 (0.16)	2.95E−04	+++	1.62E−01	6,035
3	14272	rs2853814	MT-ND6	missense	BMI	T/C	0.0012	0.0007	0.0009	0.0022	0.0012	17	0.84 (0.24)	4.90E−04	++	6.49E−01	13,636
4	14353	–	MT-ND6	–	WHR	T/C	0.9916	0.0032	0.9880	0.9978	0.0084	120	−0.33 (0.09)	2.29E−04	--	8.00E−01	14,315
5	14584	–	MT-ND6	–	BMI	T/C	0.9988	0.0007	0.9978	0.9991	0.0012	17	−0.84 (0.24)	4.90E−04	--	6.49E−01	13,636

Abbreviations and definitions: No, order number; Pos, MT position in bps; rsID, rsID name from NCBI dbSNP database when available; Gene, gene name or region; Annotation, role of the variants when available; Trait, one or more of seven traits studied; A1/2, the coded and non-coded alleles; Freq1 WeightAve, a weighted sample size average of allele frequency; FreqSE, standard error of allele frequency; MINFreq, a minimum allele frequency for contributing cohorts; MAXFreq, a maximum allele frequency for contributing cohorts; MAF, minor allele frequency; MAC, minor allele count, calculated as MAF × N; β(SE), beta coefficient and the corresponding standard error; p value, from single variant regression analysis; Dir, direction sign of contributing cohort's beta; Het-p, heterogeneity p value test from METAL; N, individuals' sample contributing in a particular marker meta-analysis (all results are of Pan-Ancestry).

and by selecting the terms "Known mitochondrial" and "Predicted mitochondrial." In MitoMiner, we kept only genes that were present in human nomenclature. MitoMiner included also the mitochondrial originated genes, which were later removed to keep our MT-nDNA list only of nuclear origin. Finally, three additional genes were added from a publication on MT-defects associated with β-cell dysfunction in a T2D mouse.[40]

MT-nDNA Candidate Genes Analysis

As a result of the above work, a list of 2,282 MT-nDNA candidate gene labels (Table S30) were used to identify any significant results from 20 GWAS papers full summary results for seven metabolic traits, representing 31 datasets (Tables 4, S4, and S6). For the adipose traits, the GWAS publication full summary results used were for BMI[41–50] and for WHR,[43,44,47–52] and the summary results data were retrieved from the GIANT Consortium repositories (see Web Resources). For glucose metabolism we used the summary results of glucose,[53–56] insulin,[54–59] HOMA-B,[54] and HOMA-IR.[54] These summary results data were retrieved from MAGIC Consortium archives (see Web Resources). For HbA1c we used two resources.[60,61] The published GWASs have large sample sizes, with a BMI study having a maximum of 339K individuals, WHR having 320K, glucose and insulin having 52K and 45K, respectively, HOMA-B and -IR having 46K, and HbA1c having 160K. Consequently, we obtained results for 109 MT-nDNA candidate genes, accompanied by 588 sentinel significant SNVs (one best per gene and trait combination, out of seven traits, Tables 4, S6, and S9).

To identify the 588 SNVs, a pre-specified selection process was followed. First, we downloaded the latest dbSNP of NCBI reference data (batch 150), and we assigned each SNV to a gene (pseudogenes excluded). The intergenic variants were assigned to the closest gene, up to half the distance between two genes. Then, we merged the 31 full-GWAS sets to the annotated dbSNP, thus each GWAS-SNP was assigned to a gene. Each summary result was merged with MT-nDNA gene labels (Table S30). The corresponding significant SNVs results, ones with the smallest p value, per gene and per trait, were accepted in the final list ($p < 0.05/(2{,}282 \times 7) = 3.13E{-}06$). After this selection, we also performed an analysis based on 1000 Genomes to identify the number of independent SNVs within a gene.[36,62] The analysis produced a mean of independent SNVs per gene of 59, median of 38, and with a maximum of two outliers 542 (*WWOX* [MIM: 605131]) and 499 (*FHIT* [MIM: 601153]). If it was a gene-based test, then a conservative threshold $p \leq 0.05/(542 \times 7 \text{ traits}) \leq 1.3E{-}05$ could have been used, which is larger than the threshold we used, $p \leq 1E{-}06$). Furthermore, to compare whether our MT-nDNA genes pass the genome-wide threshold of $p \leq 5E{-}08$, generally used in GWAS publications, we merged our gene data with all possible reported SNVs from the GWAS-Catalog (accessed 01.27.2018, Table S12 and presented findings in the Results and Discussion paragraphs).

Annotation, Enrichment Analysis, and Gene Expression and Regulation

The mtDNA as well as MT-nDNA significant variants were annotated to NCBI dbSNP build 150 (HG38); genes and their protein biological functions were annotated to NCBI Entrez Gene, GeneCards and UniProtKB; enrichment analyses were performed with MetaCore and Literature Lab; pathways with KEGG and Reactome; gene expression and regulation were assessed using

Table 2. Results of mtDNA Gene-Based Meta-analysis SKAT T1 and T5 Test ($p \leq 0.01$)

Trait	Ancestry	T	Gene	p	Qmeta	cMAF	nSNVs
HOMA-B	PA	0.01	MT-TF	5.0E−03	4604374	0.022	5
HOMA-B	PA	0.05	MT-TV	8.0E−03	3341460	0.078	6
HbA1c	EA	0.05	MT-TG	9.0E−03	13674145	0.064	13
BMI	PA	0.05	MT-TQ	1.0E−02	21201188	0.075	12
HOMA-B	PA	0.05	MT-RNR1	1.0E−02	69787232	1.323	119

Abbreviations and definitions: Trait, the trait used for a specific test; PA, Pan ancestry; EA, European ancestry; T, MAF-value threshold for selecting SNVs to be included in the gene-based association test; Gene, gene name from mtDNA; p, p value from the SKAT test; Qmeta, the SKAT Q statistics; $Q = \sum_j w_j^2 U_j^2$, where w is a weight for SNV_j and U_j is associated score statistics; cMAF, cumulative minor allele frequency; nSNVs, the number of SNVs used in the gene-based meta-analysis.

HaploReg, RegulomeDB, GTEx, and Human Protein Atlas; and protein interactions were assessed using STRING and NCBI summary of interactions from other databases (see Web Resources), with references to databases BIND, BioGRID, EcoCyc, HIV-1-human protein interaction data, and HPRD. Specifically, for GTEx gene expression eQTL analysis, the eSNVs were considered significant when the eSNV had a GTEx $p < 1E{-}07$ and was in high LD ($r^2 \geq 0.80$) with the best eSNV of the target gene. Depending on $r^2 \geq 0.80$ to 1, we called them similar to "lead" or "lead" eSNVs. Otherwise, if the LD r^2 was < 0.02 to the target gene's best eSNV, we called them "secondary" eSNVs (Table S8). Details of the resources and the corresponding references are provided in the Supplemental Material and Methods, sections 8 and 9. We have cited throughout the manuscript the corresponding gene MIM number from the Online Mendelian Inheritance in Man (see Web Resources).

Results

We evaluated the associations of mtDNA variants with seven key metabolic traits in meta-analyses of 45 cohorts (with up to N∼170,202). For details on the harmonization of the phenotypes and genotypes, see Material and Methods and Supplemental Material and Methods, sections 1–6. The Supplemental Study Descriptions and Table S1 with BMI mean and standard deviation values give a depiction of each contributing cohort in this study.

mtDNA Single-Variants Associations

Seven SNVs, two variants with average weighted MAF > 1% (Tables 1 and S2.a and Figures S1–S7) and five with MAF < 1% (Tables 1 and S2.b) displayed statistically significant evidence of association with six metabolic traits (Bonferroni threshold $p \leq 5E{-}04$, see in Material and Methods: mtDNA Meta-Analyses). MT-8706 in *MT-ATP6* (MIM: 516060) (see Web Resources), associated with WHR (with sample weighted average of cohorts' MAF = 3.24%, $p = 4.1E{-}04$) and MT-16320 (rs62581338) of the *D-loop* (with sample weighted average of cohorts' MAF = 1.58%, $p = 7.6E{-}05$) associated with fasting glucose. The five rare variants were MT-8896 (rs202120082, missense) in *MT-ATP6* associated with WHR, MT-14124 in *MT-ND5* (MIM: 516005) associated with fasting plasma insulin, MT-14272 in *MT-ND6* (rs2853814 [MIM: 516006], missense) associated with BMI, MT-14353 in *MT-ND6* associated with WHR, and MT-14584 in *ND6* associated with BMI.

The evidence of association by cohort is reported in Tables S2.a, S2.b, and S3. Typically, the cohort with a large sample size displayed the strongest evidence of association. For example, the HCHS/SOL study contributed disproportionately to several mtDNA-trait associations (see in the Supplemental Study Descriptions, CHARGEmtDNA+ Study Description). Even further, for the *MT-ATP6* (position 8706) association with WHR, the strongest association in HCHS/SOL was in those of Central/South American background (so more Native American ancestry as compared to African American ancestry, Tables S2.a and S3).

mtDNA Rare Variants Gene-Based Associations

The mitochondrial rare variants for gene-based analysis were mapped to the start and end positions of each gene from the NCBI Reference Sequence GenBank: NC_012920.1 (see Web Resources). The rare variant gene-based meta-analysis using SKAT did not yield any significant associations. In contrast, the Burden test yielded a significant association between rare variants in *MT-ND3* and BMI ($p = 1E{-}03$, $T < 0.05$ including 82 SNVs). A forest plot representing the 82 SNVs and the overall MT-ND3-overall meta is shown in Figure S17. Several gene-HOMA-B, HbA1c, and BMI associations were suggestively significant employing both the SKAT and Burden approaches (MT-RNAs *MT-TF* [MIM: 590070], *MT-TV* [MIM: 590105], *MT-TG* [MIM: 590035], *MT-TQ* [MIM: 590030], and *MT-RNR1* [MIM: 561000]; $p \leq 1E{-}02$; Tables 2 and 3). A total of 131 (T5-test) and 123 (T1-test) low-frequency and/or rare variants contributed to T5-test and T1-test, respectively. We used two gene-based approaches, burden test and sequence kernel association test (SKAT), to evaluate the association between a gene and a phenotype. Burden tests access the cumulative effects of multiple variants by assuming that all variants have the same directionality in a genetic region. SKAT tests use a score-based variance component framework without assuming that all variants have the same directionality. Although the two methods are different, they all evaluate aggregate effects of multiple low-frequency and/or rare variants

Table 3. Results of mtDNA Gene-Based Burden T1 and T5 Meta-Analysis Test ($p \leq 0.01$)

Trait	Ancestry	T	Gene	p	Beta	SE	cMAFUsed	nSNVs
BMI	PA	0.05	MT-ND3	1.0E−03	0.007	0.002	0.290	82
BMI	PA	0.05	MT-TQ	2.0E−03	0.020	0.006	0.075	12
BMI	PA	0.05	MT-CO2	5.0E−03	0.003	0.001	0.491	155
HOMAB	PA	0.01	MT-TF	5.0E−03	0.038	0.014	0.022	5
HOMAB	PA	0.01	MT-TV	6.0E−03	0.088	0.032	0.009	2
HOMAB	PA	0.05	MT-RNR1	7.0E−03	0.002	0.001	1.323	119
HOMAIR	EA	0.05	MT-TG	7.0E−03	0.027	0.010	0.061	13
BMI	EA	0.05	MT-ND3	7.0E−03	0.006	0.002	0.194	81
HOMAB	PA	0.05	MT-TV	8.0E−03	0.031	0.011	0.078	6
BMI	EA	0.05	MT-TQ	8.0E−03	0.018	0.007	0.064	12
HOMAB	EA	0.05	MT-RNR1	9.0E−03	0.002	0.001	1.287	120
HOMAB	EA	0.05	MT-TF	9.0E−03	0.018	0.007	0.127	10
HOMAB	EA	0.05	MT-TV	9.0E−03	0.030	0.011	0.079	6
HOMAB	EA	0.01	MT-RNR1	1.0E−02	0.003	0.001	0.292	61

Abbreviations and definitions: Trait, the trait used for a specific test; PA, Pan ancestry; EA, European ancestry; T, MAF-value threshold for selecting SNVs to be included in the gene-based-burden association test; Gene, gene name from mtDNA; p, p value from the gene-based burden test; the score test of the weighted sum of genotypes has the form of statistic, $T = \sum_j w_j U_j$, where w is a weight for SNV_j and U_j is the score statistic for SNV_j; Beta and SE are result of regressing the trait on a weighted sum of genotypes; cMAFUsed, cumulative minor allele frequency; nSNVs, the number of SNVs used in the gene-based burden meta-analysis.

with a phenotype. A significant gene-test reflects an aggregate effect of multiple variants in the same area. If most variants in *ND6* are not associated with the phenotype, the gene-based test might not be significant. Although statistical significance was not achieved, the association of MT-ND6 with BMI was suggestive in the burden (p = 0.06 [T5] and 0.04 [T1]) and SKAT (p = 0.15 [T5] and 0.08 [T1] tests).

Identification of MT-nDNA Candidate Variants Associated with Metabolic Traits

We identified 2,282 MT-nDNA candidate genes (see Material and Methods) and assessed their association from 20 GWASs with 31 datasets (Table S4). From the MT-nDNA candidate list, 109 genes reached statistical significance following correction for multiple testing (Bonferroni p < 1E−06) of which 46 were associated with BMI, 2 with extreme obesity, 26 with WHR, 18 with glucose, 7 with insulin, 1 with HOMA-B, 1 with HOMA-IR, and 20 with HbA1c, totaling 121 associations (Table 4). The use of additional SNVs belonging to the same 109 MT-nDNA genes, but now sourced from the GWAS catalog for the same 7 traits or any other traits, indicated that 84% (27% improvement) of MT-nDNA genes passed p ≤ 5E−08, while 16% remained at p ≤ 1E−06 without passing the p ≤ 5E−08 threshold. Of the MT-nDNA associations, *GCK* (MIM: 138079), *MRPL33* (MIM: 610059), *PPARG* (MIM: 601487), *SLC2A2* (MIM: 138160), *AMBRA1* (MIM: 611359), *NR1H3* (MIM: 602423), *MTCH2* (MIM: 613221), *IGF1* (MIM: 147440), and *BCL2* (MIM: 151430) were associated with more than one trait (Figures 2, 3, S8, and S9). For example, the well-known *GCK* is a member of hexokinases that phosphorylates glucose to produce glucose-6-phosphate, the first step in most glucose metabolism pathways, and has pleiotropic effects (see Discussion).

Enrichment Analysis of MT-nDNA Candidate Genes

The 109 MT-nDNA selected genes are candidates for MT function based on their protein localization in mitochondria, as well as from mining the published literature (Table S5). We used Literature Lab and MetaCore software and the corresponding databases to perform enrichment analyses, which provided information for MT-nDNA gene-label relations with terms, pathways, diseases, gene ontology processes, and clustering (see Supplemental Material and Methods, section 8 and Figure S10). When comparing the 109 MT-nDNA candidate genes versus the remaining 2,173 (2,282 − 109), the 109 set showed enrichment (p = 1.7E−12) for "Signal transduction, Neuropeptide signaling pathways," which included, among others, *POMC* (MIM: 176830) and *MC4R* (MIM: 155541), while no such enrichment was found for the 2,173 set. Out of 109 MT-nDNA candidate genes, 21 of the significantly associated genes were functionally related with cholesterol (Table S6 and Figure S11), 13 with glucose and insulin, and 5 with adipose/obesity. Of the 109 MT-nDNA genes, 13 associated with the "Type 2 diabetes"[54,61,63] term (Table S7) while 18 were associated with "Cardiovascular disease"[64–77] (Table S7). (For space limitation, a detailed enrichment analysis is provided in the Supplemental Material and Methods, section 8.) These

Table 4. GWAS Findings for Seven Traits (BMI, WHR, Glucose, Insulin, HOMA-B, HOMA-IR, HbA1c) for 109 MT-nDNA Candidate Genes

No	SNV	Chrom	Position	Role	Gene	Full Gene Name (Cytogenetic Position)	BMI p	OBESITY p	WHR p	GLUC p	INS p	HOMA-B p	HOMAIR p	HbA1c p
1	rs622798	1	45549599	upstream-2 kb	AKR1A1	Aldo-Keto Reductase Family 1 Member A1 (1p34.1)	8.56E−07	–	–	–	–	–	–	–
2	rs1280316	1	66843725	Intron	WDR78	WD repeat domain 78 (1p31.3)	6.81E−07	–	–	–	–	–	–	–
3	rs1093013	1	75634658	Intron	SLC44A5	Solute carrier family 44 member 5 (1p31.1)	–	–	4.86E−17	–	–	–	–	–
4	rs6428792	1	119114244	Intron	WARS2	Tryptophanyl TRNA Synthetase 2, Mitochondrial (1p12)	–	–	7.95E−18	–	–	–	–	–
5	rs2301453	1	172389027	Intron	DNM3	Dynamin 3 (1q24.3)	–	–	4.38E−17	–	–	–	–	–
6	rs4844390	1	207761504	Intron	CD46	CD46 Molecule, Complement Regulatory Protein (1q32.2)	–	–	–	–	–	–	–	6.90E−07
7	rs11118296	1	219408638	Intron	LYPLAL1	Lysophospholipase Like 1 (1q41)	–	–	3.00E−07	–	–	–	–	–
8	rs6713865	2	23676937	Intron	KLHL29	Kelch like family member 29 (2p24.1)	–	–	–	–	–	–	–	4.39E−13
9	rs934778	2	25166355	Intron	POMC	Proopiomelanocortin (2p23.3)	7.15E−07	–	–	–	–	–	–	
10	rs1107238	2	26235376	Intron	HADHA	Hydroxyacyl-CoA Dehydrogenase/3-Ketoacyl-CoA Thiolase/Enoyl-CoA Hydratase (Trifunctional Protein), Alpha Subunit (2p23.3)	8.86E−07	–	–	–	–	–	–	
11	rs13404446	2	27296386	intron	TRIM54	Tripartite Motif Containing 54 (2p23.3)	–	–	–	3.09E−09	–	–	–	–
12	rs4665965	2	27313513	intron	MPV17	MPV17, Mitochondrial Inner Membrane Protein (2p23.3)	–	–	–	1.83E−09	–	–	–	–
13	rs3736594	2	27772914	intron	MRPL33	Mitochondrial Ribosomal Protein L33 (2p23.2)	–	–	–	*3.02E−13*	*4.67E−07*	–	–	–
14	rs4346434	2	43992607	intron	LRPPRC	Leucine Rich Pentatricopeptide Repeat Containing (2p21)	–	–	8.52E−08	–	–	–	–	–
15	rs16843390	2	209655109	intron	MAP2	Microtubule Associated Protein 2 (2q34)	–	–	–	3.32E−08	–	–	–	–
16	rs715	2	210678331	utr-3-prime	CPS1	Carbamoyl-Phosphate Synthase 1 (2q34)	5.81E−07	–	–	–	–	–	–	–
17	rs933994	2	218785893	intron	CYP27A1	Cytochrome P450 Family 27 Subfamily A Member 1 (2q35)	8.65E−07	–	–	–	–	–	–	–
18	rs17036328	3	12348985	intron	PPARG	Peroxisome Proliferator Activated Receptor Gamma (3p25.2)	*4.27E−07*	–	–	–	*3.59E−12*	–	–	–

(*Continued on next page*)

Table 4. ***Continued***

No	SNV	Chrom	Position	Role	Gene	Full Gene Name (Cytogenetic Position)	BMI p	OBESITY p	WHR p	GLUC p	INS p	HOMA-B p	HOMAIR p	HbA1c p
19	rs3729931	3	12585017	intron	RAF1	Raf-1 Proto-Oncogene, Serine/Threonine Kinase (3p25.2)	–	–	3.60E−10	–	–	–	–	–
20	rs11715915	3	49417897	nc-transcript	AMT	Aminomethyltransferase (3p21.31)	–	–	–	4.90E−08	–	–	–	–
21	rs12489828	3	52532998	intron	NT5DC2	5′-Nucleotidase Domain Containing 2 (3p21.1)	–	–	2.60E−10	–	–	–	–	–
22	rs2365389	3	61250788	intron	FHIT	Fragile Histidine Triad (3p14.2)	3.75E−15	–	–	–	–	–	–	–
23	rs332375	3	66377079	intron	SLC25A26	Solute Carrier Family 25 Member 26 (3p14.1)	–	–	7.33E−07	–	–	–	–	–
24	rs1735536	3	128377770	intron	EEFSEC	Eukaryotic Elongation Factor, Selenocysteine-TRNA Specific (3q21.3)	2.95E−10	–	–	–	–	–	–	–
25	rs9844666	3	136255374	intron	PCCB	Propionyl-CoA Carboxylase Beta Subunit (3q22.3)	7.22E−07	–	–	–	–	–	–	–
26	rs11924648	3	171000207	intron	SLC2A2	Solute Carrier Family 2 Member 2 (3q26.2)	–	–	–	*1.02E−17*	–	–	–	*4.05E−09*
27	rs10012946	4	6291623	intron	WFS1	Wolframin ER Transmembrane Glycoprotein (4p16.1)	–	–	–	4.17E−07	–	–	–	–
28	rs10518406	4	122841742	intron	FGF2	Fibroblast Growth Factor 2 (4q28.1)	6.50E−07	–	–	–	–	–	–	–
29	rs1458758	4	122914724	intron	NUDT6	Nudix Hydrolase 6 (4q28.1)	–	–	6.22E−08	–	–	–	–	–
30	rs303084	4	123145793	intron	SPATA5	Spermatogenesis associated 5 (4q28.1)	–	–	3.40E−07	–	–	–	–	–
31	rs12654264	5	75352778	intron	HMGCR	3-Hydroxy-3-Methylglutaryl-CoA Reductase (5q13.3)	1.81E−08	–	–	–	–	–	–	–
32	rs10478424	5	119453325	intron	HSD17B4	Hydroxysteroid 17-Beta Dehydrogenase 4 (5q23.1)	–	–	1.40E−07	–	–	–	–	–
33	rs2881156	5	135812973	intron	SLC25A48	Solute carrier family 25 member 48 (5q31.1)	–	8.10E−07	–	–	–	–	–	–
34	rs3828870	6	16743066	intron	ATXN1	Ataxin 1 (6p22.3)	–	–	–	–	–	–	–	5.52E−07
35	rs1800562	6	26092913	intron	HFE	Hemochromatosis (6p22.2)	–	–	–	–	–	–	–	4.67E−28
36	rs1800629	6	31575254	upstream-2KB	TNF	Tumor necrosis factor (6p21.33)	–	–	7.30E−07	–	–	–	–	–
37	rs6457796	6	34860776	intron	UHRF1BP1	UHRF1 binding protein 1 (6p21.31)	1.15E−09	–	–	–	–	–	–	–

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Table 4. ***Continued***

No	SNV	Chrom	Position	Role	Gene	Full Gene Name (Cytogenetic Position)	BMI p	OBESITY p	WHR p	GLUC p	INS p	HOMA-B p	HOMAIR p	HbA1c p
38	rs10434	6	43785475	utr-3-prime	VEGFA	Vascular endothelial growth factor A (6p21.1)	–	–	8.80E−07	–	–	–	–	–
39	rs1049354	6	88143732	utr-3-prime	CNR1	Cannabinoid receptor 1 (6q15)	9.57E−07	–	–	–	–	–	–	–
40	rs9400239	6	108656460	intron	FOXO3	Forkhead box O3 (6q21)	1.61E−08	–	–	–	–	–	–	–
41	rs1273733	6	121131419	downstream-500B	TBC1D32	TBC1 domain family member 32 (6q22.31)	3.96E−12	–	–	–	–	–	–	–
42	rs1049349	6	121449496	utr-3-prime	GJA1	Gap junction protein alpha 1; synonymous: CX43 (6q22.31)	4.18E−15	–	–	–	–	–	–	–
43	rs1293954	6	151669826	intron	ESR1	Estrogen receptor 1 (6q25.1-q25.2)	4.41E−09	–	–	–	–	–	–	–
44	rs1203576	7	40808233	intron	SUGCT	*C7orf10* (Succinyl-CoA:Glutarate-CoA Transferase, 7p14.1)	1.48E−10	–	–	–	–	–	–	–
45	rs2908289	7	44184343	intron	GCK	Glucokinase (7p13)	–	–	–	*3.32E−88*	–	*6.00E−09*	–	*2.24E−19*
46	rs1088867	7	44705214	intron	OGDH	Oxoglutarate dehydrogenase (7p13)	7.90E−08	–	–	–	–	–	–	–
47	rs16892421	8	106499705	intron	OXR1	Oxidation resistance 1 (8q23.1)	–	–	6.94E−07	–	–	–	–	–
48	rs7835803	8	120030213	intron	DEPTOR	DEP domain containing MTOR interacting protein (8q24.12)	–	–	–	4.97E−07	–	–	–	–
49	rs2777795	9	104910084	intron	ABCA1	ATP binding cassette subfamily A member 1 (9q31.1)	–	–	3.13E−08	–	–	–	–	–
50	rs7023913	9	128255683	downstream-500B	DNM1	Dynamin 1 (9q34.11)	–	7.30E−07	–	–	–	–	–	–
51	rs3829109	9	136362314	intron	DNLZ	DNL-Type Zinc Finger (9q34.3)	–	–	–	1.13E−10	–	–	–	–
52	rs1244497	10	7838019	intron	TAF3	TATA-box binding protein associated factor 3 (10p14)	1.84E−11	–	–	–	–	–	–	–
53	rs5030913	10	69246375	intron	HKDC1	Hexokinase domain containing 1 (10q22.1)	–	–	–	–	–	–	–	3.56E−13
54	rs4745982	10	69330087	intron	HK1	Hexokinase 1 (10q22.1)	–	–	–	–	–	–	–	2.87E−65
55	rs7899106	10	85651147	intron	GRID1	Glutamate Ionotropic Receptor Delta Type Subunit 1 (10q23.2)	2.96E−08	–	–	–	–	–	–	–
56	rs7917772	10	102727686	intron	SFXN2	Sideroflexin 2 (10q24.32)	–	–	1.45E−09	–	–	–	–	–
57	rs1004467	10	102834750	intron	CYP17A1	Cytochrome P450 Family 17 Subfamily A Member 1 (10q24.32)	1.18E−07	–	–	–	–	–	–	–

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Table 4. ***Continued***

No	SNV	Chrom	Position	Role	Gene	Full Gene Name (Cytogenetic Position)	BMI p	OBESITY p	WHR p	GLUC p	INS p	HOMA-B p	HOMAIR p	HbA1c p
58	rs3740390	10	102878723	intron	AS3MT	Arsenite Methyltransferase (10q24.32)	4.82E−08	–	–	–	–	–	–	–
59	rs4758633	11	219538	intron	SIRT3	Sirtuin 3 (11p15.5)	–	–	–	–	–	–	–	3.44E−10
60	rs757110	11	17396930	missense	ABCC8	ATP Binding Cassette Subfamily C Member 8 (11p15.1)	4.23E−07	–	–	–	–	–	–	–
61	rs10767664	11	27704439	intron	BDNF	Brain Derived Neurotrophic Factor (11p14.1)	5.53E−13	–	–	–	–	–	–	–
62	rs11038913	11	46538180	intron	AMBRA1	Autophagy and beclin 1 regulator 1 (11p11.2)	–	–	–	*4.29E−08*	*4.91E−18*	–	–	–
63	rs11039149	11	47255124	intron	NR1H3	Nuclear Receptor Subfamily 1 Group H Member 3 (11p11.2)	–	–	–	*1.26E−12*	*4.13E−45*	–	–	–
64	rs7118178	11	47637583	intron	MTCH2	Mitochondrial carrier 2 (11p11.2)	*5.12E−08*	–	–	*3.84E−14*	*2.16E−29*	–	–	–
65	rs4246215	11	61796827	utr-3-prime	FEN1	Flap Structure-Specific Endonuclease 1 (11p12.2)	–	–	–	4.46E−11	–	–	–	–
66	rs174556	11	61813163	intron	FADS1	Fatty acid desaturase 1 (11q12.2)	–	–	–	7.82E−18	–	–	–	–
67	rs7943191	11	62561079	intron	EEF1G	Eukaryotic translation elongation factor 1 gamma (11q12.3)	–	–	4.36E−08	–	–	–	–	–
68	rs11231150	11	62584330	intron	TUT1	Terminal uridylyl transferase 1, U6 snRNA-specific (11q12.3)	–	–	5.20E−08	–	–	–	–	–
69	rs1017639	11	68831066	intron	CPT1A	Carnitine Palmitoyltransferase 1A (11q13.3)	4.96E−10	–	–	–	–	–	–	–
70	rs1296252	11	83273268	intron	CCDC90B	Coiled-Coil Domain Containing 90B (11q13.3)	7.60E−08	–	–	–	–	–	–	–
71	rs2110073	12	6966719	intron	PHB2	Prohibitin 2 (12p13.31)	–	–	–	–	–	–	–	4.44E−08
72	rs7311050	12	7013532	intron	LPCAT3	Lysophosphatidylcholine Acyltransferase 3 (12p13.31)	–	–	–	–	–	–	–	8.60E−08
73	rs1049380	12	26336611	downstream-500B	ITPR2	Inositol 1,4,5-Trisphosphate Receptor Type 2 (12p11.23)	–	–	1.42E−13	–	–	–	–	–
74	rs2408955	12	48105348	upstream-2KB	PFKM	Phosphofructokinase, muscle (12q13.11)	–	–	–	–	–	–	–	1.42E−15
75	rs35767	12	102481791	missense	IGF1	Insulin Like Growth Factor 1 (12q23.2)	–	–	–	–	*7.27E−08*	–	*7.57E−08*	–
76	rs4766578	12	111466567	intron	ATXN2	Ataxin 2 (12q24.12)	2.85E−07	–	–	–	–	–	–	1.84E−07
77	rs9581856	13	27451478	upstream-2KB	MTIF3	Mitochondrial Translational Initiation Factor 3 (13q12.2)	1.03E−08	–	–	–	–	–	–	–

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Table 4. ***Continued***

No	SNV	Chrom	Position	Role	Gene	Full Gene Name (Cytogenetic Position)	BMI p	OBESITY p	WHR p	GLUC p	INS p	HOMA-B p	HOMAIR p	HbA1c p
78	rs1124607	13	27921083	intron	PDX1	Pancreatic And Duodenal Homeobox 1 (13q12.2)	–	–	–	4.49E−07	–	–	–	–
79	rs1325363	13	33192439	intron	STARD13	StAR related lipid transfer domain containing 13 (13q13.1-q13.2)	7.25E−08	–	–	–	–	–	–	–
80	rs1078892	13	40563883	intron	FOXO1	Forkhead Box O1 (13q14.11)	5.11E−08	–	–	–	–	–	–	–
81	rs7143963	14	102838088	intron	TRAF3	TNF Receptor Associated Factor 3	2.82E−07	–	–	–	–	–	–	–
82	rs12908437	15	98744146	intron	IGF1R	Insulin Like Growth Factor 1 Receptor	–	–	–	6.32E−07	–	–	–	–
83	rs740862	16	3639677	intron	DNASE1	Deoxyribonuclease 1	3.21E−07	–	–	–	–	–	–	–
84	rs151181	16	28479196	intron	CLN3	CLN3, Battenin	2.10E−07	–	–	–	–	–	–	–
85	rs8055138	16	28880144	intron	ATP2A1	ATPase Sarcoplasmic/ Endoplasmic Reticulum Ca^{2+} Transporting 1	8.17E−17	–	–	–	–	–	–	–
86	rs749767	16	31113086	downstream-500B	BCKDK	Branched Chain Ketoacid Dehydrogenase Kinase	1.21E−09	–	–	–	–	–	–	–
87	rs7186084	16	68782357	intron	CDH1	Cadherin 1	–	–	–	–	–	–	–	1.09E−07
88	rs1847591	16	78908913	intron	WWOX	WW domain containing oxidoreductase	–	–	9.99E−07	–	–	–	–	–
89	rs9904685	17	1352101	intron	YWHAE	Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein epsilon	6.27E−07	–	–	–	–	–	–	–
90	rs4646404	17	17516885	intron	PEMT	Phosphatidylethanolamine N-Methyltransferase	–	–	5.30E−11	–	–	–	–	–
91	rs9914988	17	28856086	intron	ERAL1	Era like 12S mitochondrial rRNA chaperone 1	–	–	–	–	–	–	–	2.77E−11
92	rs242559	17	45948522	intron	MAPT	Microtubule Associated Protein Tau	–	–	–	8.29E−07	–	–	–	–
93	rs1319247	17	63106279	intron	TANC2	Etratricopeptide repeat, ankyrin repeat and coiled-coil containing 2	–	–	6.02E−08	–	–	–	–	–
94	rs12940622	17	80641771	intron	RPTOR	Regulatory Associated Protein Of MTOR Complex 1	2.49E−09	–	–	–	–	–	–	–
95	rs1044661	17	82943144	intron	B3GNTL1/ TBCD	UDP-GlcNAc:BetaGal Beta-1,3-N-Acetylglucosaminyltransferase Like 1/Tubulin Folding CofactorD			–	–	–	–	–	1.74E−46
96	rs1788785	18	23562376	intron	NPC1	NPC Intracellular Cholesterol Transporter 1	1.98E−08	–	–	–	–	–	–	–

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Table 4. ***Continued***

No	SNV	Chrom	Position	Role	Gene	Full Gene Name (Cytogenetic Position)	BMI p	OBESITY p	WHR p	GLUC p	INS p	HOMA-B p	HOMAIR p	HbA1c p
97	rs17066842	18	60373391	upstream-2KB	MC4R	Melanocortin 4 Receptor	6.40E−14	–	–	–	–	–	–	–
98	rs12454712	18	63178651	intron	BCL2	BCL2, Apoptosis Regulator	–	–	*1.10E−09*	–	*1.39E−07*	–	–	–
99	rs757318	19	18709498	intron	CRTC1	CREB Regulated Transcription Coactivator 1	8.76E−09	–	–	–	–	–	–	–
100	rs2075650	19	44892362	intron	TOMM40	Translocase Of Outer Mitochondrial Membrane 40	1.25E−08	–	–	–	–	–	–	–
101	rs405509	19	44905579	upstream-2 kb	APOE	Apolipoprotein E	2.65E−07	–	–	–	–	–	–	–
102	rs2281361	20	32140338	intron	TM9SF4	ransmembrane 9 superfamily member 4	9.78E−07	–	–	–	–	–	–	–
103	rs878639	20	35306660	intron	UQCC1	Ubiquinol-Cytochrome C Reductase Complex Assembly Factor 1	–	–	1.50E−11	–	–	–	–	–
104	rs2076574	20	41092733	intron	TOP1	DNA topoisomerase I	–	–	4.11E−07	–	–	–	–	–
105	rs5750373	22	37028990	intron	MPST	Mercaptopyruvate Sulfurtransferase	–	–	–	–	–	–	–	2.17E−07
106	rs2284099	22	43155830	intron	TSPO	Translocator Protein	–	–	6.65E−07	–	–	–	–	–
107	rs1050828	23	154536002	missense	G6PD	Glucose-6-phosphate dehydrogenase	–	–	–	–	–	–	–	8.23E−135
108	rs1448032	23	155052530	intron	FUNDC2	FUN14 domain containing 2	–	–	–	–	–	–	–	5.92E−17
109	rs5940514	23	155559972	intron	TMLHE	Trimethyllysine hydroxylase, epsilon	–	–	–	–	–	–	–	2.22E−19

p values in italics annotate SNVs that associate as significant with more than one trait.

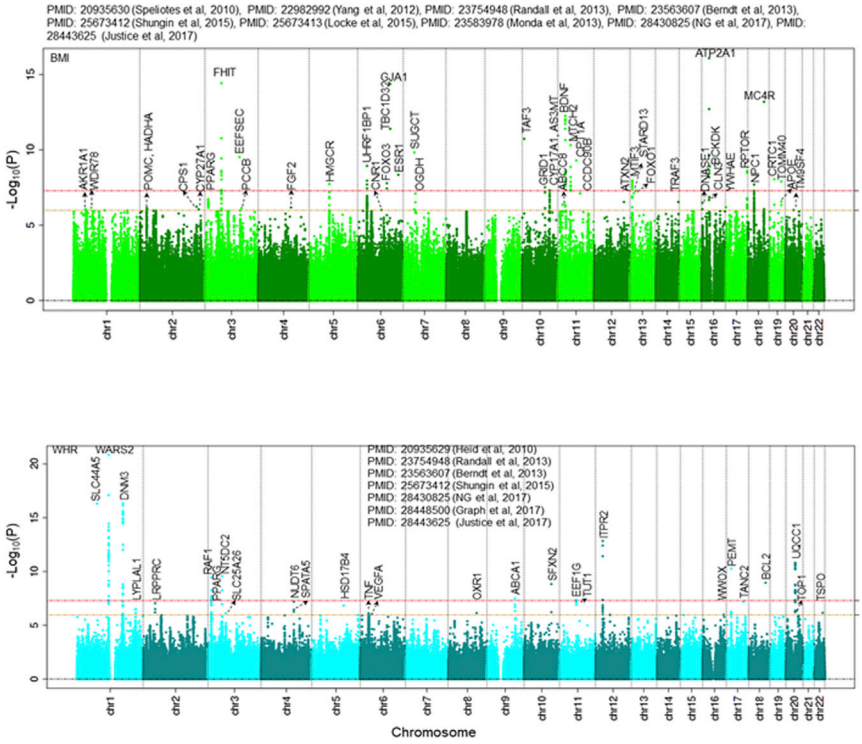

Figure 2. BMI and WHR Association Results with MT-nDNA Candidate Genes

findings demonstrate the importance of several MT-nDNA candidates to cardiometabolic outcomes.

eQTLs of MT-nDNA Candidate Variants

Several variants in or near MT-nDNA candidate genes were identified as expression QTL (eSNV) (Table S8). Based on RegulomeDB,[78] three variants were the best in eSNVs features' ranking. The first was rs242559, intronic to *MAPT* (MIM: 157140). Mutations in *MAPT* associate with lower mitochondrial nicotinamide adenine dinucleotide (NADH) levels,[79] partially suppress complex I-driven respiration, and lower overall ATP production by oxidative phosphorylation, with cells relying on glycolysis to maintain ATP levels. The second, rs9897919, is a 3′ UTR variant for *TBCD* (MIM: 604649), tubulin folding cofactor D, and *B3GNTL1* (17q25.3 [MIM: 615337]), a putative glycosyltransferase. The third, rs1788821, is intronic to *NPC1* (MIM: 607623), which is an intracellular cholesterol transporter with a role in the egress of cholesterol from the endosomal/lysosomal compartments (Table S9).

The findings of RegulomeDB were reinforced by HaploReg (v.4.1).[80] The MT-nDNA variants showed an enrichment in transcription regulation features. For instance, rs242559 of *MAPT* is localized within the promoter histone marks in skeletal muscle, at enhancer histone marks of 16 tissues, and at DNase marks of 4 tissues. In addition, the rs242559 polymorphism alters the protein binding site of GATA2, a transcription factor protein that binds in the promoter regions of target genes (Table S9).

To determine the eSNVs' gene targets in specific tissues, we used GTEx (v.7.0)[81,82] with a summary in Table S8 and detailed in Table S10. The 42 unique eSNVs were assigned to 29 genes targeting regulation of 50 genes, distributed among 13 tissues (adipose, tibial artery, thyroid, skin, blood, brain, skeletal muscle, esophageal muscularis, fibroblast, liver, pancreas, testis, and tibial nerve). There were 28 unique lead and 18 unique secondary (LD r^2 was < 0.02 to the target gene's best eSNV) eSNVs identified (see Material and Methods). For example, rs2510344 of *NPC1* regulates *C18orf8* in skin and rs11663558 of *NPC1* regulates its own *NPC1* gene expressed highest in subcutaneous adipose tissue (GTEx data).

trans-eQTLs of MT-nDNA Candidate Variants

We combined the GWAS p value for the MT-nDNA SNVs with additional evidence from *trans*-gene expression regulation for a specific variant using GWAS3D[83] (Figures S12.1–S12.3). The GWAS3D software selected 16 cell types, which included chromosomal looping data (5C or ChIA-PET or Hi-C) and important transcriptional marker data (H3K4me1, H3K27ac, DHSs, EP300, and CTCF).[83] Among several *trans*-eQTLs, for example, rs2881156 ($p = 8.1E{-}07$) of *SLC25A48* (5q31.1 [MIM: 616150], Figure S12.1) associated with obesity and *trans*-regulated expression of three genes: *SAR1B* (5q31.1 [MIM: 607690]) involved in protein transport from the endoplasmic reticulum to the Golgi (mutations in this gene are a cause of chylomicron retention disease [MIM: 246700]);[84] *TRPC7* (5q31.1), a regulator of intracellular calcium levels;[85] and *REEP2* (5q31.2 [MIM: 609347]), which enhances the function of sweet taste receptors[86] and is about 80 times higher expressed in brain than in other tissues (GTEx data).

PPI Network

We analyzed 109 MT-nDNA proteins using the PPI network (see Web Resources)[87,88] to identify 4,132 interacting proteins. We present the 15 top genes (Table S11) with highest PageRank score for PPI,[89–91] including the number of PPI, a short description of gene's function from Gene Entrez of NCBI-db, associated trait(s), and association p value(s) (see also Supplemental Material and Methods, section 9). From the PPI analysis it is evident that a gene/protein hub (which is assumed important because of a relatively large number of interactions) is not necessary, the top-notch for association with a specific trait, as shown in the Discussion section.

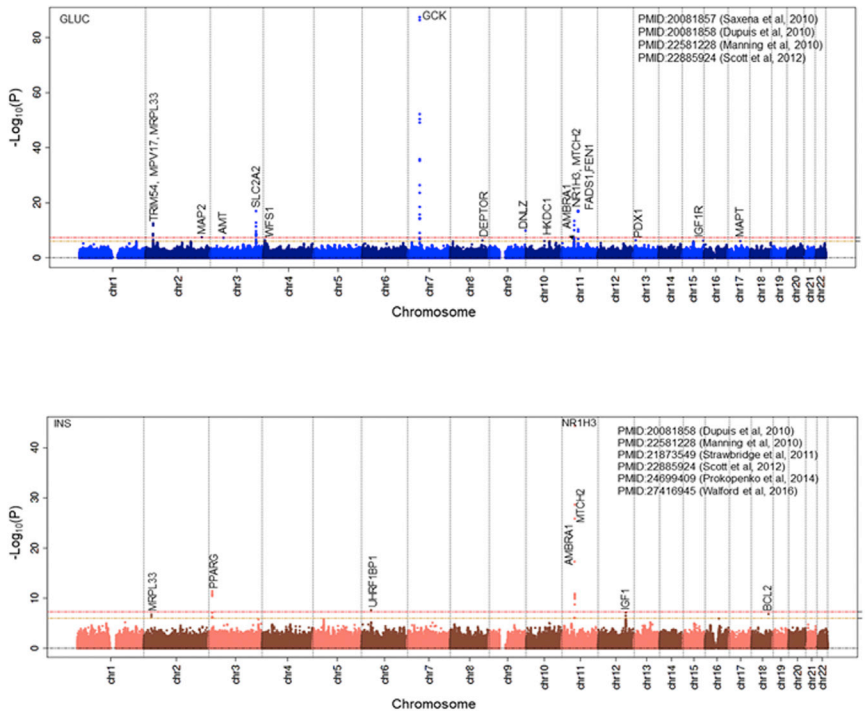

Figure 3. Glucose and Insulin Association Results with MT-nDNA Candidate Genes

Discussion

This large, comprehensive study of human mitochondrial and nuclear mitochondrial genetic variation in relation to seven key risk factors for metabolic disease provides important information toward a better understanding of the causes and mechanisms of these phenotypes. The use of a two-tiered approach—i.e., examining mtDNA variants for up to ~170,202 individuals and nuclear MT-nDNA candidate gene polymorphisms for up to ~339,000 individuals—facilitated the evaluation of the genetic underpinnings of seven metabolic traits reflecting adiposity/obesity and glucose-insulin metabolism and signaling. We identified two mtDNA SNVs (in *MT-ATP6* and the *D-loop*) associated with WHR and fasting glucose, a burden of SNVs in a gene (*MT-ND3*) associated with BMI, and five rare SNVs of mtDNA (in *MT-ATP6*, *MT-ND5*, and *MT-ND6*) associated with BMI (2 of 3), with WHR (2 of 3), and with fasting insulin (1 of 3). Additionally, the MT-nDNA meta-analysis implicated significant associations with 62 of 109 protein coding candidate genes ($p \leq 5E{-}08$), 29 additional passing the above threshold for the metabolic traits considered herein or any other traits querying the GWAS catalog (see Web Resources). Only 16% of MT-nDNA findings passed $p \leq 1E{-}06$ but did not reach $p \leq 5E{-}08$, and thus may be considered as new MT-nDNA contributions in association with seven metabolic traits (Tables 4 and S12). Enrichment analysis showed that 13 of MT-nDNA genes have also been identified as candidate genes for T2D risk and 18 for CVD risk. The eQTL analysis of MT-nDNA candidates revealed that approximately 27% of associations with seven metabolic traits also act as eSNVs in regulating expression of other *cis*-genes. In addition, *trans*-eQTL analysis yielded potential support for maternal and X chromosome epigenetic gene expression regulation. The PPI network analysis identified top PageRank-ed interacting genes from our significant MT-nDNA associations, but they did not necessarily represent the highest trait-specific associations. Overall, these findings indicate the important role of both the nuclear genome and mtDNA-related variants in energy production and, in turn, in the polygenic architecture of metabolic traits. Below we summarize and discuss our most salient study findings.

mtDNA and Metabolic Disease Associations

To date, nearly 290 genetic causes for rare disorders of mitochondrial energy generation have been identified.[92] These rare mitochondrial genetic diseases commonly result in multiple clinical phenotypes with varying penetrance, likely due to differences in heteroplasmy.[93] In our study, *MT-ATP6* variants MT-8706 and MT-8896 (rs202120082), one with MAF > 1% and the other one rare, respectively, were both associated with WHR. *MT-ATP6* is part of the proton-transporting portion of ATP synthase, contributing to its rotational mechanism.[94,95] rs202120082 (MT-8896) is a missense mutation (p.Ala124Thr) and conversion of the hydrophobic alanine to an uncharged, polar threonine at this site may have implications for ATP6's ability to effectively pump protons and thus decrease production of ATP energy units.

MT-16320 (rs62581338; MAF > 1%), in the *D-loop*, which contains a few mtDNA replication origins and is possibly involved in accelerating mtDNA synthesis to satisfy developmental, physiological, or aging-related demands,[96] displayed a significant association with glucose. MT-16320 is less than 1 kb from a major mtDNA transcription initiation site (IT) that overlaps with promoters (in the D-loop in H-strand, IT_{H1} at 561 bps and IT_{H2} at 630, and in L-strand at 407 bps (IT_{L1}))[97] and is 500 bps downstream of mitochondrial-encoded cytochrome b (*MT-CYB* [MIM: 516020], plus strand) and 2 kb upstream of *MT-ND6* (minus strand).

Of the five rare SNVs, MT-14272 (rs2853814, missense), MT-14353, and MT-14584 of *MT-ND6* were associated with adiposity-related traits. *MT-ND6* is a core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (complex I) and is part of the minimal assembly required for catalysis. Complex I functions in the transfer of electrons from NADH into the respiratory chain, while

coupling the flow of electrons to the pumping of protons. We also demonstrated an association between insulin and rare variant MT-14124 in the closely related *MT-ND5* gene. *MT-ND5* is also a core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (complex I). Future studies need to validate these rare variant mtDNA findings.

It is also important to note that we observed several rare gene-based suggestive associations between our metabolic traits ($p \leq 5E{-}02$, Tables 2 and 3) and non-protein coding genes, primarily tRNAs. Mutations within tRNAs that impact translation would impact all of the mtDNA-encoded peptides and consequently would be expected to have significant effects on oxidative phosphorylation. The *MT-ND3* gene displayed an aggregate of low-frequency variants associated with BMI. *MT-ND3* is a subunit of the respiratory chain complex I that is part of the minimal assembly of core proteins required to catalyze NADH dehydrogenation and electron transfer to ubiquinone (coenzyme Q10). Interestingly, previous studies have demonstrated increased *MT-ND3* gene expression associated with a higher histological severity of hepatic steatosis.[98] Gene-based meta-analysis burden test, which employed inverse variance weighting to combine association results from 82 variants in MT-ND3 gene across cohorts, yielded a significant association between MT-ND3 and BMI ($p = 1E{-}03$, Figure S17). This figure illustrates that rare alleles of 82 SNVs contributing in the MT-ND3 meta-analysis have variable effects.

We used GTEx data for mtDNA genes as assembled by the Human Protein Atlas team (see Web Resources). They showed the highest expression of RNA for the *MT-ATP6*, *MT-ND3*, *MT-ND5*, and *MT-ND6* significant mtDNA genes in the heart and brain, which represent the highest energy-demanding tissues as compared, for example, to adipose tissue, in a ratio of about 3:1 (Figure S13). The importance of MT-nDNA and mtDNA gene polymorphisms in the heart is supported by recent publications that have summarized the relation of MT to vascular function,[99] as therapeutic targets in heart failure,[100] and specific genes and pathways that relate to the heart.[101,102]

MT-nDNA Candidate Genes Associations (Glycemic Traits)

Glucose sensing in β cells is largely controlled by the hexokinase proteins. Three of the genes encoding hexokinases, *HK1* (MIM: 142600), *HKDC1* (MIM: 617221), and *GCK*, were part of our MT-nDNA candidate list and were found to be significantly associated with glucose, HOMA-B, and/or HbA1c (Table 4). Hexokinases catalyze the conversion of glucose into glucose-6-phosphate. The phosphorylation of glucose directly couples extra-mitochondrial glycolysis to intra-mitochondrial oxidative phosphorylation. *GCK*, for example, with pleiotropic effects, produces an enzyme in the pancreas which plays a role in glucose-stimulated insulin secretion and affects glucose uptake and conversion to glycogen in the liver. The *GCK* GWAS-identified variant rs2908289 (Table 4) has been previously associated with glucose, HOMA-B, HbA1c,[54,60] and other variants in LD with it, more recently associated with BMI (rs4607517, $r^2 = 0.65$, $p = 8E{-}56$),[55] with T2D (rs1799884, $r^2 = 0.81$, $p = 5E{-}18$),[103] and with metabolic syndrome (rs3757840, $r^2 = 0.18$, $p = 4E{-}13$).[104] Mitochondrial metabolism, which drives the respiratory chain to produce ATP via oxidative phosphorylation, also contributes to glucose sensing, since disruption of mitochondrial oxidative metabolism blocks glucose-stimulated insulin secretion.[40,105–107] Our analysis shows that 13 additional MT-nDNA candidate genes are annotated with SNVs that associate with glucose/insulin metabolism (Figure S11). Diabetes mellitus has been associated with maternally inherited mutations in *MT-TK* (MIM: 590060), *MT-TS2* (MIM: 590085), and *MT-TE* (MIM: 590025), where the molecular mechanisms involve impaired translation of mtDNA-encoded proteins.[3] In addition, MT-nDNA candidate genes *POLG* (15q26.1 [MIM: 174763], DNA polymerase gamma); *RRM2B* (8q22.3 [MIM: 604712], a ribonucleotide reductase); *OPA1* (3q29 [MIM: 605290], a nuclear-encoded mitochondrial protein with similarity to dynamin-related GTPases); and *MPV17* (2p23.3 [MIM: 137960], a mitochondrial inner membrane protein) have been associated with diabetes mellitus, largely due to an impairment in mtDNA maintenance.[108] In our study, rs1050828, a missense mutation of *G6PD* (Xq28 [MIM: 305900], 154536002 bps; $p = 8.2E{-}135$) was associated with HbA1c. *G6PD* catalyzes the rate-limiting step of oxidative pentose-phosphate pathways, a route for dissimilation of carbohydrates besides glycolysis. It contributes to the production of NADPH and pentose phosphatases for fatty acid and nucleic acid synthesis (Table S6 and Figure S14). *G6PD* plays an essential role in maintaining health by protecting against oxidative damage.[109,110] The same rs1050828 variant of G6PD has previously been reported as associated with HbA1c lowering in African Americans but not in other populations, leading to misdiagnosis/under-diagnosis of T2D.[61] In the last century, a number of papers reported a maternal excess transmission of T2D,[111–113] suggesting the possibility of an epigenetic transmission of diabetes mediated by the mother. To date, two genes, *KLF14* (7q32.2 [MIM: 609393])[114] and *KCNQ1* (11p15.5-p15.4 [MIM: 607542]),[115,116] have been described as potential candidates that show parent-of-origin effects in association with T2D. However, based on our MT-nDNA list, these two genes are not MT-nDNA candidates. In our study, GWAS3D analysis identified *G6PD* as *trans*-regulated with the expression of *MECP2* (Xq28 [MIM: 300005]) (Figure S12.3).[117] *MECP2* binds to methylated DNA and mediates transcriptional repression through interaction with histone deacetylase and the corepressor *SIN3A* (15q24.2 [MIM: 607776]) (Figure S14). Lai et al.[118] reported meta-results that T2D-affected case subjects with *G6PD* deficiency had two times higher odds of developing diabetes than unaffected control subjects.[119] Furthermore,

G6PD protein has a weak score of 1 out of 5 to be found in MT. G6PD is mainly localized in the cytosol and also to the microtubule-organizing center and vesicles (Human Protein Atlas). G6PD protein has 69 interactions and was ranked 65th by the PageRank algorithm for the importance of PPI. These results taken together may suggest that *G6PD* contributes to the maternal epigenetics of T2D, through X chromosome inheritance and less through MT. Other candidates of MT-nDNA genes that may contribute to mitochondrial epigenetics[120] are *DNMT1* (19p13.2 [MIM: 126375], DNA methyltransferase 1), *POLRMT* (19p13.3 [MIM: 601778], RNA polymerase mitochondrial), *TFB1M* (6q25.3 [MIM: 607033], transcription factor B1, mitochondrial), *TFB2M* (1q44 [MIM: 607055], transcription factor B2, mitochondrial), and *TFAM* (10q21.1 [MIM: 600438], transcription factor A, mitochondrial), but showed no significant associations with existing data for the traits studied (for BMI: rs6926853, *TFB1M*, p = 1.3E−03;[45] WHR: rs10465617, *TFB2M*, p = 3.1E−03;[43] glucose: rs4804124, *DNMT1*, p = 7.3E−05;[54] insulin: rs7253062, *DNMT1*, p = 4.4E−03;[55] HOMA-B: rs12462004, *DNMT1*, p = 4.4E−03;[54] HOMA-IR: rs892189, *DNMT1*, p = 7E−04;[54] HbA1c: rs11006132, *TFAM*, p = 2.4E−03[61]).

MT-nDNA Candidate Genes Associations (Adipose Traits)

We found that a total of 26 of the 109 MT-nDNA candidate genes showed significant associations with WHR (Tables 4 and S6 and Figure S11). For example, *WARS2* (1p12 [MIM: 604733]) encodes the MT tryptophanyl-tRNA synthase. We observed an intronic rs6428792 of *WARS2* in association with WHR (p = 7.95E−18).[50] Another SNV, rs10923724 in LD with rs6428792, r^2 = 0.29, WHR p = 9E−25)[52] upstream of *WARS2* but closer to downstream of *TBX15* (1p12 [MIM: 604127]), modifies *WARS2* expression in skeletal muscle (p = 1.1E-36) and in adipose-subcutaneous tissues (p = 1E−s29) (GTEx data). In our PPI analysis of 109 MT-nDNA genes and 4,132 interactants, *WARS2* showed two interactions and was ranked 2,028th based on the PageRank algorithm. Taken together, these findings (our study, GTEx, and GWAS) suggest that associations with WHR might be mediated also by differential expression of *WARS2*.[121]

There were 48 MT-nDNA candidates associating with BMI. For example, *NPC1* (18q11.2, Tables 4 and S6) has been associated with early childhood onset and adult morbid obesity.[122] *NPC1* (a cholesterol transporter) is highly expressed in human white adipose tissue adipocytes with increased levels in obese individuals.[123] Our lead SNV rs1788785 is an eSNV for *NPC1* and is not in LD with the best *NPC1* eSNV, supporting *NPC1* as a candidate gene for obesity. Indeed, studies in mice have shown that a non-functioning *NPC1* resulted in late-onset weight loss and less food intake.[124] In humans, *NPC1* is part of the cholesterol metabolism pathway (see Web Resources). Recently, *NPC1* mutant cells were reported to have fragmented mitochondrial networks, increased respiration, alterations in the composition of the respiratory chain complex, and a substantial reduction in the cellular ATP level. Thus, a primary lysosomal defect in *NPC1* mutant fibroblasts is accompanied by deregulation of the organization and function of the mitochondrial network.[125] *NPC1* was ranked 241st and had 13 interactions in the PPI network.

Other notable obesity-related variants/genes among MT-nDNA candidates include *POMC* (2p23.3, adrenocorticotropic peptide/hormone, p = 7.2E−07), which binds to melanocortin 2 receptor (*MC2R*, 18p11.21 [MIM: 607397]), stimulating release of cortisol, a steroid stress-response hormone. *MC4R* (18q21.32, p = 6.4E−14) was ranked the 3rd in BMI-effects compared to *FTO* (16q12.2 [MIM: 610966]), the top ranked for BMI effects in Speliotes et al.,[41] Locke et al.,[46] and Winkler et al.[48] *MC4R* is another member of the melanocortin receptor family, which has a central role in energy homeostasis and somatic growth. *MC4R* is ranked the 82nd, with 12 PPI, when analyzed for the importance of PPI using the PageRank algorithm. *CNR1* (6q15 [MIM: 114610], cannabinoid receptor, p = 9.6E−07) influences mitochondrial respiration. *CRTC1* (19p13.11 [MIM: 607536], CREB regulated transcription coactivator 1, p = 8.8E−09) is a potent coactivator of *PGC1a* (4p15.2, officially known as PPARGC1A [MIM: 604517]), a transcriptional coactivator that regulates the genes involved in energy metabolism, and inducer of mitochondrial biogenesis.

MTCH2 (11p11.2, mitochondrial carrier homolog 2, rs7118178, BMI p = 5.1E−08) was 9th in BMI effects compared to *FTO* in Speliotes et al.[41] and 16th in Locke et al.[46] MTCH2 is located in the inner membrane of MT, highly expressed in white adipose tissue and adipocytes, and is thought to play a regulatory role in adipocyte differentiation and biology.[126] The *MTCH2* GWAS-variant rs7118178 (Table 4) has been associated with BMI,[46] glucose,[36,55] and pro-insulin[58] (Figure 4, T1-4). *MTCH2* has 87 protein interactions and ranked 14th when analyzed for the importance of PPI using PageRank algorithm. *MTCH2* deficiency in mouse muscle has been shown to be beneficial, protecting mice from the obesogenic effect of high-fat diets, most likely the result of an increase in mitochondrial metabolism. *MTCH2* is proposed as a repressor of muscle mitochondrial metabolism and size.[127] Using both GWAS and eQTL information, three *MTCH2* SNVs co-localize within a 15 kbp DNA region. Based on GTEx data, rs4752856 in *MTCH2* (Figure 4, T6) regulates gene expression of *C1QTNF4* (11p11.2 [MIM: 614911]) and *PSMC3* (11p11.2 [MIM: 186852]) in subcutaneous adipose tissue. rs3817335, which is in high LD (r^2 = 0.903) with rs4752856, regulates *SLC39A13* (11p11.2 [MIM: 608735]) in tibial artery tissue, while rs7118178 (in less LD to two other SNVs, r^2 = 0.083) regulates *CELF1* (11p11.2 [MIM: 601074]) in tibial nerve tissue, and with results similar to the best eSNVs for the mentioned genes and in high LD with their eSNVs (Figure 4, T5). *C1QTNF4* is reported as a potential cytokine

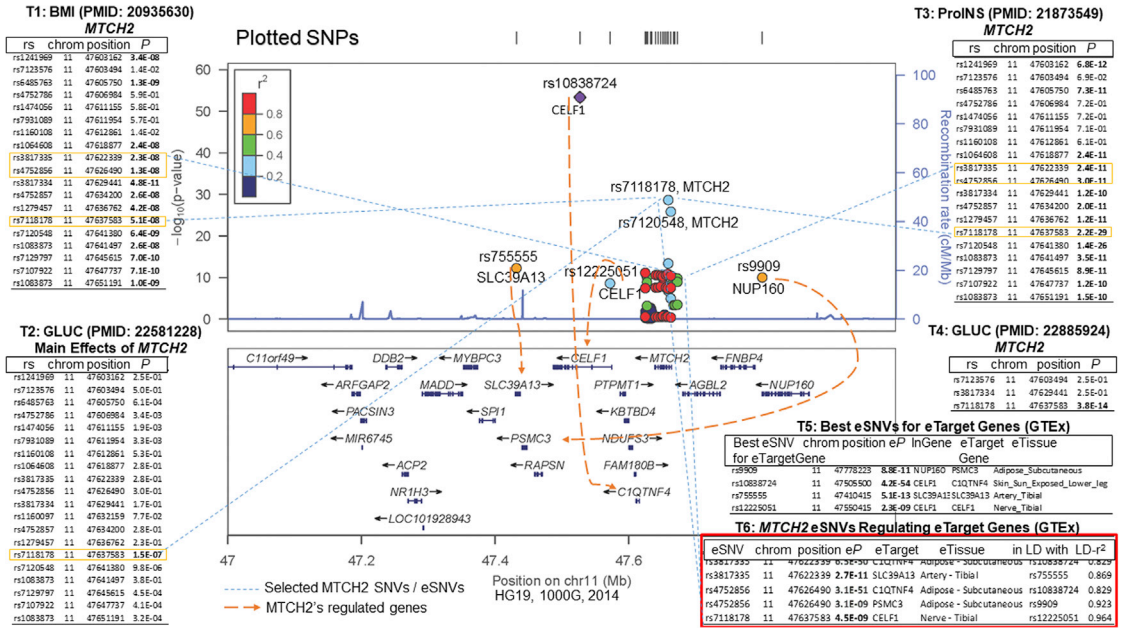

Figure 4. Selected SNVs of *MTCH2* Significantly Associating with BMI, Glucose, Insulin, and a Few of Them Regulating the Expression (eSNVs) of at Least Four Genes: *C1QTNF4, SLC39A13, PSMC3,* and *CELF1*

First, we used LocusZoom[142] for plotting regional information for *MTCH2* selected SNVs, which have been reported by large GWAS publications (for BMI[41] [T1], for glucose[55] [T2], for proinsulin[58] [T3], and another large analysis for glucose[56] [T4]), for strength and extent of the association signal relative to genomic position, local linkage disequilibrium, and recombination patterns and the positions of genes in the region. Second, we used GTEx[81] data to identify the SNVs of *MTCH2* that are eSNVs by influencing the expression of other *cis*-target-genes (*C1QTNF4*, *PSMC3*, *SLC39A13*, *CELF1*) (T6). Third, we verified whether these eSNVs of *MTCH2* (connected with blue dashed lines) are true expression regulators, by identifying the best eSNVs of the four target genes (T5, regulation effects shown with orange-dashed lines) and evaluating the LD r^2 between the *MTCH2* eSNV, e.g., rs4752856 (T6) and the best eSNV rs10838724 (T5) for the corresponding target gene (*C1QTNF4*), which was found to be 0.829 in adipose-subcutaneous tissue (T6). Fourth, the *MTCH2* SNVs serving as eSNVs for the four *cis*-target-genes, shown in T6, colocalize in about 15K bps region. Thus, *MTCH2* associates with BMI, glucose, and proinsulin, and among others influence the gene expression of other *cis*-genes (*C1QTNF4*, *PSMC3*, *SLC39A13*, *CELF1*), who in itself contribute also to individuals' obesity risk (see Discussion).

that can induce the activation of both *NFKB1* (MIM: 164011) and *IL6/STAT3* (MIM: 147620/102582) signaling pathways[128] and acts in the hypothalamus to modulate food intake and peripheral energy expenditure.[129] *PSMC3* is involved in ATP-dependent degradation of misfolded and damaged proteins and in removal of no longer required proteins. This protein is also involved in cell cycle progression, apoptosis, and DNA damage repair. A knockdown of *PSMC3* in human immortalized fibroblasts increased cell proliferation.[130] *CELF1* has been associated with Alzheimer disease and obesity.[131] Consequently, *MTCH2* SNVs regulate gene expression of other *cis*-genes related to obesity, food intake, and/or cell number. Thus, *MTCH2* effects on BMI may be more complex than previously accounted for.

Other Functions of MT-nDNA Candidate Genes

Mitochondrial inheritance has been previously associated with obesity, metabolic syndrome, insulin resistance, type 2 diabetes, and cardiovascular disease.[3,40,132–135] The complex genetics of MT affects processes of glucose, lipid, and amino acid catabolism, with ATP as the final energy product. By-products of these processes play an important role in signaling (e.g., ROS) and are used for epigenetic modifications (e.g., acetyl CoA). In our study, we would expect a preponderance of glycemic and lipid genes and proteins. Out of 109 MT-nDNA candidate genes, 21 of the significantly associated genes were functionally related with cholesterol (Table S6 and Figure S11), 16 with glucose and insulin, and 5 with adipose/obesity. The 109 MT-nDNA candidate genes have other functions too. For example, rs1044661 is located at 17q25.3, overlapping two genes that run in opposite strands: *TBCD* (involved in tubulin folding as cofactor D) and *B3GNTL1* (a putative glucosyltransferase). This SNV was associated with HbA1c ($p = 1.74E{-}46$).[61] Recently, Francis et al.[136] hypothesized that the interaction between *TBCD* (17q25.3) and *ARL2* (11q13.1 [MIM: 601175]) suggest that *ARL2* serves as regulator of both mitochondrial fusion and microtubule dynamics. In 109 MT-nDNA candidates, *WDR78* (1p31.3), *TRIM54* (2p23.3 [MIM: 606474], regulating titin kinase [controlling elasticity in muscle] and microtubule-dependent signaling pathways in striated muscles), *DNM3* (1q24.3 [MIM: 611445], involved in vesicular transport and with biased expression in brain), *MAP2* (2q34 [MIM: 157130], involved in microtubule assembly and neurogenesis), *GJA1* (6q22.31 [MIM: 121014], involved in the

contraction of heart) and *MAPT* (17q21.31, expressed in nervous system) produce microtubule-associated proteins in interaction with MT (Table S6 and Figure S11). It should be noted that rs1050828, a missense mutation of *G6PD* residing on X chromosome, may impact HbA1c through non-glycemic factors rather than glucose metabolism.[61] Additional functions of MT-nDNA candidate genes are described in the Supplemental Material and Methods, section 7.

Strengths and Limitations

Our study had many strengths. We used large sample sizes for studying mtDNA and MT-nDNA. At the mtDNA level we validated SNVs used per cohort and imputed based on Cosmopolitan MT-1000 Genomes. At the MT-nDNA level we used the strength of many consortia contributions for associations with seven traits. As a consequence, we performed comprehensive mtDNA and MT-nDNA analyses. The seven mtDNA variants significant associations showed no statistical significant deviations from the homogeneity/similarity of beta contributions before meta-pooling across studies as reported with Het-p in Table 1, as well as graphically represented in the Forest plot (Figure S16). For supporting MT-future studies, we have provided mtDNA updated Tables S15–S28 for arrays used in this study including MitoMap annotation. Also, we include Table S32 of predictions from the bioinformatics platforms that have been shown to have the highest sensitivity and specificity for the mtDNA variants.[137] Our study also had some limitations. Rare mtDNA mutations are usually heteroplasmic point mutations and/or mtDNA lesions that typically result in a primary mitochondrial disease, which can manifest as a broad range of clinical outcomes. In this study we have interrogated the effect of inherited mtDNA variants, much commoner in the population than typical mtDNA mutations. Therefore, while there is a great deal of literature linking inherited mtDNA variants to disease, many of these studies have limited statistical power[138] and several have never been independently replicated. So in many respects there is not a great deal of robust literature to call upon. The harmonization of mtDNA in each study was dependent on the number of mtDNA variants and their quality. Some variants were dropped before performing any imputation, because low-quality variants matched not only to the mtDNA genome, but also to nuclear genome (see mtDNA Variant Harmonization in Material and Methods). A number of quality-control filters were applied to mtDNA, which reduced individual sample size per marker or the number of useful markers. We did not address the association of significant markers by gender, because in our study sample sizes per mtDNA marker were variable and often small.

Perspective and Conclusion

We focused on associations of MT with adipose and glycemic traits, yet we acknowledge that there is still much to be learned from other traits, for example for triglycerides, high-density lipoprotein cholesterol, and C-reactive protein.[46,139–141] Notably, we identified 21 MT genes of lipid metabolism, although this was not the direct focus of our study. For example, *TMLHE* (Xq28 [MIM: 300777], trimethyllysine dioxygenase associated with HbA1c, $p = 2.2E{-}19$) is the first enzyme in the carnitine biosynthesis pathway. Carnitine play an essential role in the transport of activated fatty acids across the inner mitochondrial membrane and this gene is ubiquitous for its expression in heart. Hence, MT associations with other traits at the consortia-level remain to be explored. In conclusion, we identified common, rare SNVs, and a gene burden of genetic mtDNA variants, as well as 109 MT-nDNA genes associated with metabolic traits. Of the 109 MT-nDNA candidate genes, a subset pointed to associations with adipose, glucose metabolism, T2D, and CVD, and a subset of SNVs was inferred to contribute for differential regulatory genes expression. We documented in this study that the MT-candidates (SNVs/genes) have special contributions for functioning and energy homeostasis in adipose tissues and to glucose metabolism and insulin signaling.

Accession Numbers

Summary statistics for the mtDNA meta-analyses are available in the dbGaP repository for the CHARGE Consortium, phs000930.v8.p1.

Supplemental Data

Supplemental Data include 17 figures, 32 tables, Supplemental Material and Methods, Supplemental Acknowledgments, and Supplemental Study Descriptions and can be found with this article online at https://doi.org/10.1016/j.ajhg.2018.12.001.

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Declaration of Interests

A.Y.C. is currently an employee of Merck & Co. C.T.-P. has received grants from Bayer pertaining to antithrombotic treatment of atrial fibrillation and cardiac valve replacements. D.O.M.-K. works as a part-time clinical research consultant for Metabolon, Inc. The remaining authors declare no competing financial interests.

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Web Resources

1000 Genomes data (released April 2016), http://ftp.1000genomes.ebi.ac.uk/vol1/ftp/release/20130502/
dbGaP, https://www.ncbi.nlm.nih.gov/gap
EasyQC, http://www.genepi-regensburg.de/easyqc/
FCGENE, https://sourceforge.net/projects/fcgene/
GenBank (NCBI Reference Sequence: NC_012920.1), https://www.ncbi.nlm.nih.gov/nuccore/251831106
GIANT Consortium repositories, https://portals.broadinstitute.org/collaboration/giant/index.php/GIANT_consortium_data_files
GTEx (Gene Expression Portal), https://gtexportal.org
GWAS Catalog, http://www.ebi.ac.uk/gwas/
Human Mitochondrion Sequence, https://www.mitomap.org/MITOMAP/HumanMitoSeq
IMPUTE2, http://mathgen.stats.ox.ac.uk/impute/impute_v2.html
KEGG, https://www.genome.jp/dbget-bin/www_bget?hsa04979
MAGIC consortium archives, https://www.magicinvestigators.org/downloads/
OMIM, http://www.omim.org/
Protein-Protein Interactions, ftp://ftp.ncbi.nih.gov/gene/GeneRIF/
seqMeta, https://github.com/DavisBrian/seqMeta
seqMeta release notes, https://cran.r-project.org/web/packages/seqMeta/seqMeta.pdf
SHAPEIT2, https://mathgen.stats.ox.ac.uk/genetics_software/shapeit/shapeit.html#home
The Human Protein Atlas, http://www.proteinatlas.org/

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